



---

Year: 2013

---

## **Nicotinamide phosphoribosyltransferase/visfatin expression by inflammatory monocytes mediates arthritis pathogenesis**

Présumey, Jessy ; Courties, Gabriel ; Louis-Plence, Pascale ; Escriou, Virginie ; Scherman, Daniel ; Pers, Yves-Marie ; Yssel, Hans ; Pène, Jérôme ; Kyburz, Diego ; Gay, Steffen ; Jorgensen, Christian ; Apparailly, Florence

**Abstract:** OBJECTIVES: Nicotinamide phosphoribosyltransferase (NAMPT)/pre-B-cell colony-enhancing factor/visfatin exerts multiple functions and has been implicated in the pathogenesis of rheumatoid arthritis. To gain insight into its role in arthritis and given that NAMPT is identified as a novel mediator of innate immunity, we addressed the function of monocyte-derived NAMPT in experimental arthritis by selective gene knockdown in inflammatory monocytes. METHODS: siRNA uptake and NAMPT expression were determined in Ly6C(high) and Ly6C(low) monocyte subsets following intravenous injection of siRNA against NAMPT (siNAMPT) or non-targeting siRNA (siCT) formulated with the DMAPAP cationic liposome into mice. Mice with established collagen-induced arthritis (CIA) were treated weekly after disease onset with siNAMPT or siCT and clinical features were assessed. T-helper cell frequencies, cytokine production and percentage of IL-6-producing Ly6C(high) monocytes were analysed. Using a co-culture system consisting of purified CD14 monocytes and autologous CD4 T cells, NAMPT and cytokine production, and the percentage of IL-17-producing CD4 T cells, were determined following transfection of CD14 monocytes with siCT or siNAMPT. RESULTS: On intravenous injection, siRNA was preferentially engulfed by Ly6C(high) monocytes, and siRNA-mediated silencing of NAMPT expression in Ly6C(high) monocytes inhibited CIA progression. This effect was associated with reduced IL-6 production by Ly6C(high) monocytes, reduced proportion of Th17 cells and autoantibody titers, and decreased activation and infiltration of monocytes/macrophages and neutrophils in arthritic joints. Moreover, NAMPT-RNAi-silenced CD14 monocytes were found to reduce the percentage of IL-17-producing CD4 T cells in vitro. CONCLUSIONS: Our results show that the expression of NAMPT in Ly6C(high) monocytes promotes many downstream effects involved in inflammatory arthritis and demonstrate the utility of targeting disease-causing genes, such as NAMPT, in Ly6C(high) monocytes for therapeutic intervention in arthritis.

DOI: <https://doi.org/10.1136/annrheumdis-2012-202403>

Posted at the Zurich Open Repository and Archive, University of Zurich  
ZORA URL: <https://doi.org/10.5167/uzh-76667>  
Journal Article

Originally published at:

Présumey, Jessy; Courties, Gabriel; Louis-Plence, Pascale; Escriou, Virginie; Scherman, Daniel; Pers, Yves-Marie; Yssel, Hans; Pène, Jérôme; Kyburz, Diego; Gay, Steffen; Jorgensen, Christian; Apparailly, Florence (2013). Nicotinamide phosphoribosyltransferase/visfatin expression by inflammatory monocytes mediates arthritis pathogenesis. *Annals of the Rheumatic Diseases*, 72(10):1717-1724.

DOI: <https://doi.org/10.1136/annrheumdis-2012-202403>

## **TITLE PAGE**

# **NAMPT/Visfatin expression by inflammatory monocytes mediates arthritis pathogenesis**

Jessy Pr sumey<sup>1,2,\*</sup>, Gabriel Courties<sup>1,2,\*</sup>, Pascale Louis-Plence<sup>1,2</sup>, Virginie Escriou<sup>3,4,5,6</sup>, Daniel Scherman<sup>3,4,5,6</sup>, Yves-Marie Pers<sup>1,2,7</sup>, Hans Yssel<sup>1,2</sup>, J r me P ne<sup>1,2</sup>, Diego Kyburz<sup>8</sup>, Steffen Gay<sup>8</sup>, Christian Jorgensen<sup>1,2,7</sup>, and Florence Apparailly<sup>1,2,7</sup>

<sup>1</sup>INSERM, U844, University Hospital Saint Eloi, 80 avenue Augustin Fliche, 34295 Montpellier, France

<sup>2</sup>University of Medicine, 2 Rue Ecole de M decine, 34000 Montpellier, France

<sup>3</sup>INSERM, U1022, UFR des Sciences Pharmaceutiques et Biologiques, 4 Avenue de l'Observatoire, 75006 Paris, France

<sup>4</sup>CNRS, UMR8151, 75006 Paris, France

<sup>5</sup>Chemical and Genetic Pharmacology and Imaging Laboratory, Facult  de Pharmacie, Universit  Paris Descartes, 75270 Paris, France

<sup>6</sup>Ecole Nationale Sup rieure de Chimie de Paris, 11 Rue Pierre et Marie Curie, 75005 Paris, France

<sup>7</sup>University Hospital Lapeyronie, clinical department for osteoarticular diseases, 371 Avenue Gaston Giraud, 34295 Montpellier, France

<sup>8</sup>Center of Experimental Rheumatology, University Hospital Zurich and Zurich Center of Integrative Human Physiology, 8091 Zurich, Switzerland

Corresponding author: Florence Apparailly, INSERM U844, CHU Saint Eloi, 80 rue Augustin Fliche, 34295 Montpellier cedex 5, France. Phone: +33-499-636-086, Fax: +33-499-636-020, email: [florence.apparailly@inserm.fr](mailto:florence.apparailly@inserm.fr)

\*Equal contribution to this work

Conflict of interest: The authors declare having no conflict of interest

Present affiliation and location:

Gabriel Courties: Center for Systems Biology, Massachusetts General Hospital and Harvard Medical School, 185 Cambridge Street, Boston, Massachusetts 02114, USA

Jessy Presumey: The Immune Disease Institute and Program in Molecular and Cellular Medicine, Children's Hospital and Harvard Medical School, Boston, Massachusetts 02115, USA.

## ABSTRACT

**Objectives** Nicotinamide phosphoribosyltransferase (NAMPT)/PBEF/Visfatin exerts multiple functions and has been implicated in the pathogenesis of rheumatoid arthritis. To gain insight into its role in arthritis and given that NAMPT is identified as a novel mediator of innate immunity, we addressed the function of monocyte-derived NAMPT in experimental arthritis by selective gene knockdown in inflammatory monocytes.

**Methods** siRNA uptake and NAMPT expression were determined in Ly6C<sup>high</sup> and Ly6C<sup>low</sup> monocyte subsets following intravenous injection of siRNA against NAMPT (siNAMPT) or non-targeting siRNA (siCT) formulated with the DMAPAP cationic liposome into mice. Mice with established collagen-induced arthritis (CIA) were treated weekly after disease onset with siNAMPT or siCT and clinical features were assessed. T helper cell frequencies, cytokine production and percentage of IL-6-producing Ly6C<sup>high</sup> monocytes were analyzed. Using a coculture system consisting of purified CD14<sup>+</sup> monocytes and autologous CD4<sup>+</sup> T cells, NAMPT and cytokine production, as well as the percentage of IL-17-producing CD4<sup>+</sup> T cells were determined following transfection of CD14<sup>+</sup> monocytes with siCT or siNAMPT.

**Results** Upon intravenous injection, siRNA was preferentially engulfed by Ly6C<sup>high</sup> monocytes and siRNA-mediated silencing of NAMPT expression in Ly6C<sup>high</sup> monocytes inhibited CIA progression. This effect was associated with reduced IL-6 production by Ly6C<sup>high</sup> monocytes, reduced proportion of Th17 cells and auto-antibodies titers, as well as decreased activation and infiltration of monocytes/macrophages and neutrophils in arthritic joints. Moreover, NAMPT-RNAi-silenced CD14<sup>+</sup> monocytes were found to reduce the percentage of IL-17-producing CD4<sup>+</sup> T cells in vitro.

**Conclusions** Taken together, our results show that the expression of NAMPT in Ly6C<sup>high</sup> monocytes promotes many downstream effects involved in inflammatory arthritis and demonstrate the utility of targeting disease-causing genes, such as NAMPT, in Ly6C<sup>high</sup> monocytes for therapeutic intervention in arthritis.

Keywords: inflammatory monocytes, RNA interference, NAMPT, Th17 cells, arthritis.

## INTRODUCTION

Rheumatoid arthritis (RA) is a systemic inflammatory disorder that leads to the progressive destruction of joints. Although the etiology of RA remains unknown, many cell types and molecules are implicated in the pathogenesis of RA. Monocytes/macrophages are now believed to be local and systemic amplifiers of the disease in regards to their abundance as well as their paracrine effects on other immune cells and resident fibroblasts.<sup>1</sup> The therapeutic efficacy of conventional anti-rheumatic drugs coincides with down-regulation of biological functions of monocytes/macrophages and biotherapies blocking cytokines produced predominantly by monocytes/macrophages are effective in the treatment of RA.<sup>2,3</sup> The rheumatoid synovium contains activated monocytes/macrophages that produce large amounts of inflammatory mediators, including IL-1 $\beta$ , IL-6 and TNF- $\alpha$ , which contribute to synovial cell proliferation, maintenance of an inflammatory environment and structural joint damage. Notably, this cytokinic milieu promotes the development and maintenance of Th17 cells<sup>4</sup> that play a pathogenic role in RA by linking T cell activation and bone resorption.<sup>5-7</sup> Recently, it has been demonstrated that activated monocytes continuously migrate into the inflamed joints of patients with active RA<sup>8</sup> where they acquire an activated phenotype and specifically promote Th17 cell differentiation, but not Th1 or Th2 cell responses.<sup>9</sup> Thus, excessive activity of CD14<sup>+</sup> monocytes participates to inflammatory disease progression, including RA, but the molecular pathways involved in their dysregulated function remain elusive.

Nicotinamide phosphoribosyltransferase (NAMPT) is an ubiquitously expressed protein that displays multiple functions.<sup>10,11</sup> It was originally identified as pre-B-cell colony enhancing factor (PBEF), a secreted factor that synergizes with IL-7 and stem cell factor to promote the growth of B cell precursors.<sup>12</sup> It is also proposed as visfatin, an adipokine produced by visceral fat that stimulates insulin signaling.<sup>13</sup> It may also function as an essential enzyme in

the NAD biosynthetic pathway that enhances cellular resistance to genotoxic/oxidative stress and may confer to cells of the immune system the ability to survive during stressful situations such as inflammation.<sup>14</sup> Of note, NAMPT has recently been identified as a novel mediator of innate immunity with pro-inflammatory properties involved in the persistence of inflammation.<sup>15,16</sup> NAMPT is upregulated in monocytes upon activation and induces the production of various pro-inflammatory cytokines including IL-6.<sup>17-19</sup>

NAMPT has been implicated in the pathogenesis of several human diseases including RA<sup>11</sup> and its increased expression in peripheral blood mononuclear cells is proposed as a marker of chronic inflammation.<sup>20</sup> Elevated expression of NAMPT is reported within the synovial tissue and blood of patients with RA and correlates with disease activity.<sup>17,21,22</sup> Increased expression of NAMPT is also confirmed in mice with collagen-induced arthritis (CIA), both in serum and in the inflamed paws, and early in vivo blockade of NAMPT with the small molecule inhibitor APO866 ameliorates CIA severity.<sup>21,23</sup> These findings support a critical role of NAMPT in the pathogenesis of arthritis. Nevertheless, how NAMPT expression in specific cell populations impacts the inflammatory response of arthritis remains unknown.

At least two main subsets of circulating monocytes are described in humans and rodents. In mice, Ly6C<sup>low</sup> monocytes survey endothelial cells and surrounding tissues for damage or viral infection while Ly6C<sup>high</sup> monocytes are highly phagocytic, secrete inflammatory mediators, give rise to macrophages and dendritic cells, and are involved in inflammatory disease progression.<sup>24,25</sup> Given that NAMPT is expressed by both monocyte subsets and the importance to target the Ly6C<sup>high</sup> monocytes subset for therapeutic benefit in a broad range of inflammatory disorders,<sup>26</sup> we undertook in vivo experiments to explore the impact of the RNAi-mediated silencing of NAMPT in Ly6C<sup>high</sup> monocytes in mouse CIA.



## **MATERIALS AND METHODS**

DMAPAP cationic lipid was synthesized as previously described<sup>27</sup> and mixed with DOPE and nucleic acids as previously described.<sup>28</sup>

DBA/1 mice were used in these studies, which were approved by the Ethics Committee on Animal Research of the Languedoc-Roussillon region (CE-LR-0505), and CIA was induced as previously described.<sup>29</sup> Arthritis severity was graded and analyzed as previously described.<sup>30,46</sup> Detailed procedures are available in Supplementary files.

## RESULTS

### **In vivo silencing of NAMPT in Ly-6C<sup>high</sup> monocytes**

We previously reported that the cationic liposome DMAPAP efficiently delivers siRNA to CD11b<sup>+</sup> monocytes and knockdown genes upon systemic administration.<sup>31</sup> We first examined the respective capacity of both Ly-6C<sup>low</sup> and Ly-6C<sup>high</sup> monocyte subsets to engulf siRNA lipoplexes upon intravenous injection (figure 1A). Mice were injected with a single dose of 0.5mg/kg of Cy3-labeled siRNA and the percentage of siRNA-containing cells were monitored after 24 hours according to the gating strategy described in figure S1. While less than 10% of circulating Ly-6C<sup>low</sup> monocytes engulfed the labeled siRNA (8.5%  $\pm$  1.3%), the presence of the siRNA was detected in 63%  $\pm$  7% of the Ly-6C<sup>high</sup> monocytes. Similarly, Ly-6C<sup>high</sup> monocytes from the splenic reservoir engulfed the siRNA more efficiently than Ly-6C<sup>low</sup> monocytes (30.6%  $\pm$  6.2% and 5.4%  $\pm$  1.6% respectively). To a lesser extent, the presence of siRNA was also detected in macrophages and conventional DCs from the spleen of injected animals (see supplemental figure S2A). Of note, the siRNA was not incorporated into the CD45<sup>+</sup> mononuclear cells (see supplemental figure S2B) and was taken up by less than 1% of the circulating T and B cells (see supplemental figure S2C).

We then evaluated the feasibility to silence NAMPT gene in Ly-6C<sup>high</sup> monocytes (figure 1B). Systemic injection of mice with DMAPAP-formulated siRNA sequences specific for mouse NAMPT (siNAMPT) resulted in a 66% reduction of NAMPT protein expression in Ly-6C<sup>high</sup> monocytes from blood as compared to mice injected with non-targeting control siRNA (siCT). Although NAMPT was expressed at lower levels in Ly-6C<sup>low</sup> monocytes, its expression was not affected following the administration of siNAMPT lipoplexes. In agreement with the inefficient delivery of siRNA to neutrophils (less than 2% of the

circulating or tissue Ly-6G<sup>+</sup> neutrophils), NAMPT levels were altered neither in the blood neutrophils (see supplemental figure S3A) nor in the neutrophils infiltrating arthritic joints (data not shown). Together, these data demonstrate that the cationic liposome DMAPAP efficiently delivers siRNA to the inflammatory Ly-6C<sup>high</sup> monocytes and silence NAMPT expression, as opposed to the non-inflammatory Ly-6C<sup>low</sup> monocytes and other cells of the hematopoietic system, when administered intravenously in mice.

### **RNAi-mediated silencing of NAMPT in Ly6C<sup>high</sup> monocytes impairs the development of CIA.**

Considering the importance of Ly6C<sup>high</sup> monocytes in inflammatory disorders and inflammatory properties reported for NAMPT, we investigated the effect of siNAMPT delivery to Ly-6C<sup>high</sup> monocytes in experimental arthritis. Collagen-induced arthritic (CIA) mice were injected intravenously with siNAMPT lipoplexes once a week after disease onset (figure 2). Mice treated with siNAMPT lipoplexes showed a significant improvement of disease features from the first day of treatment, as compared with control animals (figure 2A). Given that disease activity is associated with increased serum IL-6 levels, we determined the effect of NAMPT inhibition in Ly6C<sup>high</sup> monocytes on IL-6 levels by ELISA at euthanasia. The results showed that the protection from disease progression in the siNAMPT-treated group was associated with a significant decrease of circulating IL-6 levels ( $p=0.0485$ ), as compared with mice administered with siCT lipoplexes (figure 2B). High levels of circulating CII-specific IgG antibodies, characterized by a high IgG2a:IgG1 ratio, are a hallmark of CIA and determine disease severity. We showed that, consistent with improved clinical features at

euthanasia in the group of siNAMPT-treated mice, and opposed to the control group, a significant decrease of CII-specific IgG2a:IgG1 ratio was measured (figure 2C).

To further investigate the impact of the systemically delivered siRNA on joint inflammation, we determined the effect of repetitive siNAMPT lipoplex injections on cellular infiltrates. Consistent with the observed clinical benefit, the total number of cells infiltrating ankle joints of CIA mice was reduced in the siNAMPT-treated group compared with siCT-injected animals (figure 2D). Among these cells, decreased counts of neutrophils (see supplemental figure S3B), resident macrophages and inflammatory monocytes (figure 2D; see gating strategy in supplemental figure S1C) were observed. Interestingly, the production of TNF- $\alpha$  was significantly lower in the three cell types for CIA mice injected with siNAMPT lipoplexes, as compared with controls (figure 2E and S3C). These results indicate that the siNAMPT systemic treatment not only lead to a decrease in the number of immune cells infiltrating arthritic joints, but also in their activated status.

### **In vivo inhibition of NAMPT in Ly6C<sup>high</sup> monocytes induces broad immunomodulation of arthritic conditions.**

Considering that IL-6 is abundantly expressed in arthritis by a number of cell types, including monocytes/macrophages, and that NAMPT induces the production of IL-6 upon monocyte activation, we investigated whether siRNA-mediated silencing of NAMPT in Ly6C<sup>high</sup> monocytes impacts IL-6 production by this specific cell subset in vivo under inflammatory conditions. Arthritic mice were injected weekly from disease onset (arthritic score > 3) with siNAMPT lipoplexes and spleen cells collected after 3 weeks. As we previously demonstrated that the CIA development is similar between PBS- and siCT-injected mice,<sup>32,33</sup> this latter

condition was used as control in the following experiments. Flow cytometric analysis of intracellular IL-6 staining showed that in vivo silencing of NAMPT in Ly-6C<sup>high</sup> monocytes during arthritis progression significantly reduced the percentage of IL-6-producing Ly-6C<sup>high</sup> monocytes ( $6,6 \pm 0,7\%$ ), as well as the production of IL-6 by these cells ( $\text{MFI}=21,5 \pm 0,2$ ), as compared with siCT-injected mice ( $12,2 \pm 0,8\%$  and  $\text{MFI}=36,2 \pm 3,3$  respectively). The reduction of IL-6 production upon NAMPT knockdown was also confirmed by ELISA measurement of IL-6 protein levels ( $p=0,0221$ ) in 24-hours culture supernatant of spleen cells as compared with control mice (figure 3B). Analysis of pro-inflammatory cytokine profiles of these mice showed that levels of TNF- $\alpha$ , IL-17A and IFN- $\gamma$  were reduced in splenocytes of siNAMPT-treated mice compared with siCT-injected animals, while the production of the anti-inflammatory cytokine IL-10 was increased (figure 3B).

Considering that IL-6 is critical for Th17 differentiation and expansion in mouse and its expression was reduced in Ly-6C<sup>high</sup> monocytes in arthritic mice treated with siNAMPT, we hypothesized that Th17 responses might be reduced in siNAMPT-treated CIA mice. The percentage of IL-17A-producing T cells and IL-17 production were measured by intracellular cytokine staining of CD4<sup>+</sup> T cells (figure 3C). The results showed a reduced percentage of IL-17A positive T cells ( $0,8 \pm 0,1\%$  versus  $1,8 \pm 0,3\%$ ) and IL-17A production ( $\text{MFI}=24112 \pm 1832$  versus  $42881 \pm 2062$ ) in the spleen of arthritic mice treated with siNAMPT lipoplexes relative to siCT-injected animals. Interestingly, the down-regulation of the Th17 population in CIA was not due to a general effect on T cell activation because neither the Th1 response, characterized by IFN- $\gamma$ -producing CD4<sup>+</sup> T cells, nor the frequency of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells, were significantly affected (figure 3C-D). Overall, our data indicate that interfering with the NAMPT/IL-6 axis in inflammatory Ly-6C<sup>high</sup> monocytes enables the modulation of pathogenic Th17 cells and reduction of inflammatory cytokines in CIA.

### **Silencing of NAMPT in human CD14<sup>+</sup> monocytes interferes with Th17 cell expansion.**

Since one of the in vivo downstream anti-inflammatory effects of NAMPT silencing in Ly6C<sup>high</sup> monocytes is the decrease in Th17 cells, we explored in vitro whether NAMPT expression in human monocytes may also impact the Th17 cell responses. First, Cy3-labelled siRNA lipoplexes (25 nM) were added to human PBMCs to determine the uptake of the siRNA (figure 4A). The CD14<sup>+</sup>CD16<sup>-</sup> monocytes were found to overwhelmingly incorporate the siRNA (98.3%  $\pm$  0.2%), while it was only taken up by 58.4%  $\pm$  1.2% of the CD14<sup>dim</sup>CD16<sup>+</sup> monocytes. Importantly, CD14<sup>+</sup>CD16<sup>-</sup> monocytes incorporated 4 times more siRNA than the CD14<sup>dim</sup>CD16<sup>+</sup> monocytes (MFI=16.613  $\pm$  185 versus 4.535  $\pm$  272). Consistent with what we observed following in vivo administration to mice, human CD3<sup>+</sup> T and CD19<sup>+</sup> B lymphocytes did not reveal any positive signal for the presence of siRNA (data not shown). We next assessed the functional silencing in human monocytes using siNAMPT lipoplexes. Based on previously described procedures,<sup>34,35</sup> CD14<sup>+</sup> monocytes from the blood of healthy donors were transfected with either siCT or siNAMPT in the presence of LPS. NAMPT silencing was quantified at mRNA and protein levels two days after transfection. More than 50% of the NAMPT transcripts and 30% of the protein levels were down-regulated upon siNAMPT application compared with controls, as shown by RT-qPCR and flow cytometry respectively (figure 4B). Down-regulation of NAMPT expression in CD14<sup>+</sup> monocytes was associated with a significant decrease of both IL-6 and TNF- $\alpha$  expression levels (figure 4C). Next, to assess the effect of NAMPT silencing in CD14<sup>+</sup> monocytes on Th17 cell expansion, CD14<sup>+</sup> monocytes were co-cultured 48 hours after transfection with autologous purified CD4<sup>+</sup> T cells and the percentage of IL-17-producing CD4<sup>+</sup> T cells was monitored 5 days later. Addition of siNAMPT-transfected CD14<sup>+</sup> monocytes to autologous

CD4<sup>+</sup> T cells resulted in a significant decrease in the percentage of IL-17A-producing CD4<sup>+</sup> T cells, as opposed to siCT-transfected monocytes (figure 4D). These data show that inhibition of NAMPT gene expression in human CD14<sup>+</sup> monocytes reduces the expansion of Th17 cells.

## DISCUSSION

NAMPT exerts multiple functions in a variety of physiological processes and its overexpression has been implicated in the pathogenesis of a number of inflammatory disorders, including cancer, atherosclerosis, diabetes and RA.<sup>11</sup> Numerous studies have shown that NAMPT regulates inflammatory mediators and apoptosis. For instance, NAMPT protects macrophages from ER stress-induced apoptosis by activating an IL-6/Stat3 signaling pathway.<sup>32</sup> We recently reported that NAMPT is overexpressed by FLS of RA patients and stimulates the production of IL-6, IL-8, MMP-1 and MMP-3 in RASF, as well as IL-6 and TNF- $\alpha$  in monocytes.<sup>17</sup> Monocytes, a key cell type in RA pathogenesis, are themselves a source of NAMPT, which expression is up-regulated under inflammatory conditions<sup>18</sup> and able to induce IL-6 transcription in these cells,<sup>17-19,36,37</sup> further substantiating the autocrine role of NAMPT in inducing IL-6 expression by monocytes. Nevertheless, the mechanisms underlying the deleterious effect of NAMPT in the inflammatory process of RA remain unclear.

In the present study, we found that systemic administration of siRNA-containing lipoplexes able to deliver and silence NAMPT in Ly6C<sup>high</sup> monocytes ameliorated disease features in a mouse model of RA. Significant clinical benefit was demonstrated by reduced systemic and local inflammatory markers of arthritis, including reduced serum IL-6 levels, decreased anti-bCII antibody titers and marked reduction in the number of Th17 cells, as well as in the number and activation status of Ly6C<sup>high</sup> monocytes, macrophages and neutrophils infiltrating the arthritic joints. In arthritic joints, TNF- $\alpha$  is one of the most deleterious cytokine implicated in inflammatory processes, and we showed that NAMPT down-regulation in Ly6C<sup>high</sup> monocytes leads to a significant decrease of TNF- $\alpha$  expression by their main producers, i.e. monocytes and macrophages. Our in vitro and in vivo studies performed on



human CD14<sup>+</sup> and mouse Ly6C<sup>high</sup> monocytes respectively showed that one of the downstream effects of knocking down NAMPT expression in this cell subset was a decreased IL-6 expression by these cells, associated with an impaired Th17 cell expansion. We cannot exclude that NAMPT silencing may affect other genes than IL-6 and TNF- $\alpha$  and is likely to involve other mechanisms of immune modulation accounting for the observed beneficial effects. However, we can exclude that the defect of Ly-6C<sup>high</sup> monocyte recruitment observed in inflamed joints from siNAMPT-treated mice was not due to a reduced expression of the CCR2/MCP1 axis (data not shown). Interestingly, although NAMPT gene silencing in Ly-6C<sup>high</sup> monocytes was associated with a markedly reduced frequency of IL-17A-producing CD4<sup>+</sup> T cells, the numbers and percentage of Th1 and Treg cells were unchanged. Consistent with these findings, it has been reported that the administration of an anti-IL-6R suppressed arthritis and inhibited Th17 differentiation but did not alter Th1, Th2 or Treg cell levels.<sup>36,38</sup> Importantly, clinical benefits demonstrated in the trials in which RA patients were treated with the neutralizing anti-IL-6R mAb tocilizumab showed reduced IL-6 serum levels, dampened T- and B-lymphocyte-mediated inflammatory responses, and suppressed x-ray progression of disease. In conclusion, we demonstrate that NAMPT expression in Ly-6C<sup>high</sup> monocytes participates to arthritis progression, not only confirming that NAMPT plays an important role in inflammatory responses, but also suggesting that Ly-6C<sup>high</sup> monocytes are among key cellular mediators of these pro-inflammatory actions.

Rheumatoid arthritis (RA) is a chronic inflammatory disorder in which the CD14<sup>+</sup> monocytes continuously migrate into the injured joint tissue<sup>8,39,40</sup> and act as local and systemic amplifiers of disease through the multitude and abundance of their paracrine/autocrine acting mediators.<sup>1</sup> Their numbers are increased in clinically affected joints and correlate with clinical signs,<sup>41</sup> and thus strategies aiming at interfering with this specific cell subset to provide therapeutic

benefit are intensely challenged. To date, two main approaches have been used to impact on monocytes' survival or recruitment, either by their depleting or by antagonizing chemokine receptors important in their trafficking.<sup>42</sup> Although these strategies showed promising results in animal models of RA, none of them were effective in RA patients.<sup>43</sup> The present work suggests that alternative strategies might be envisioned such as silencing the expression of a master gene implicated in pro-inflammatory functions of Ly6C<sup>high</sup> monocytes to modify their activation status, their capacity to prime T and B cell responses, to activate neutrophils, and to alter overall disease progression. The present study thus indicates that strategies based on the "Trojan monocyte" for drug delivery to inflamed sites are of particular interest in arthritis, and may be applied to a broad range of inflammatory disorders.

As NAMPT is expressed in many cell types and exerts multiple functions, the latter strategy however requires overcoming the major challenges in achieving efficient *in vivo* RNAi, i.e. being able to deliver the therapeutic siRNA to those cells. Previously, we have shown that, upon intravenous administration, the cationic liposome DMAPAP formulation distributes siRNAs to the myeloid compartment.<sup>33</sup> Here, we further refined the siRNA distribution to the respective monocyte subsets and demonstrate that the siRNA is preferentially taken up by the Ly-6C<sup>high</sup> monocyte subset, as opposed to the Ly-6C<sup>low</sup> monocytes, in the blood, spleen, liver and arthritic joints (data not shown), resulting in efficient and specific silencing of NAMPT in Ly-6C<sup>high</sup> monocytes. Although to lesser extent, siRNA uptake was also detected in tissue resident macrophages and cDC, thought to arise from Ly-6C<sup>high</sup> monocytes under inflammatory conditions.<sup>44</sup> Importantly, we show here that siRNA was not efficiently taken up by non-hematopoietic cells and only very weakly incorporated into T and B lymphocytes or neutrophils (<2% of positive cells) and that no variation in NAMPT expression was evidenced in Ly-6C<sup>low</sup> monocytes. Our *in vitro* data on human PBMCs show that the liposome

formulation enables efficient delivery of siRNA to classical CD14<sup>+</sup>CD16<sup>-</sup> monocytes. To a lesser extent, the siRNA was also taken up by non-classical CD14<sup>dim</sup>CD16<sup>+</sup> monocytes. However the amount of siRNA engulfed by this subset (showed by MFI) is very low compared to CD14<sup>+</sup>CD16<sup>-</sup> monocytes. Although comparing efficiencies between in vitro and in vivo experiments is not relevant, these data suggest that different mechanisms might be involved in cationic liposome uptake by monocyte subsets, and perhaps between human and mouse monocytes, that need to be further characterized.

Several studies have demonstrated the importance of Ly6C<sup>high</sup> monocytes in the pathogenesis of inflammatory disorders. In experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis, Ly-6C<sup>high</sup> monocytes accumulate in the blood and migrate to the central nervous system (CNS) before disease onset, where they differentiate into DCs and macrophages in EAE lesions.<sup>45</sup> Moreover, increased numbers of circulating Ly-6C<sup>high</sup> monocytes are associated with enhanced disease severity. In a mouse model of myocardial infarct, Ly-6C<sup>high</sup> monocytes were found to first migrate to the site of inflammation during the acute phase.<sup>46</sup> Finally, when the accumulation of Ly-6C<sup>high</sup> monocytes in sites of inflammation was prevented by silencing CCR2 expression, a reduction of atherosclerotic lesions in apoE<sup>-/-</sup> mice and of myocardial ischemia injury was reported, as well as a prolonged islet graft survival.<sup>26</sup> Altogether, these studies underscore the contribution of Ly-6C<sup>high</sup> monocytes to pathological events in a broad range of inflammatory and autoimmune disorders. The specific implication of Ly-6C<sup>high</sup> monocyte was not however determined in arthritis. Here, using the experimental mouse CIA model, we show that Ly-6C<sup>high</sup> monocytes also play a key role in arthritis pathogenesis.

Our findings identify NAMPT as a critical gene that can be targeted to modulate pro-inflammatory cytokines produced by Ly-6C<sup>high</sup> monocytes, to impact on their crosstalk with

other immune cells and to interfere with inflammatory responses in arthritis. The downstream molecular mechanisms responsible for the anti-inflammatory effects of NAMPT-mediated silencing in Ly6C<sup>high</sup> monocytes remain however to be elucidated.

## FOOTNOTES

### Authors' email addresses :

[jessy.presumey@gmail.com](mailto:jessy.presumey@gmail.com); [gab.courties@gmail.com](mailto:gab.courties@gmail.com); [diego.kyburz@usz.ch](mailto:diego.kyburz@usz.ch); [steffen.gay@usz.ch](mailto:steffen.gay@usz.ch);  
[virginie.escrion@parisdescartes.fr](mailto:virginie.escrion@parisdescartes.fr); [daniel.scherman@parisdescartes.fr](mailto:daniel.scherman@parisdescartes.fr); [hans.yssel@inserm.fr](mailto:hans.yssel@inserm.fr);  
[jerome.pene@inserm.fr](mailto:jerome.pene@inserm.fr); [pascale.plence@inserm.fr](mailto:pascale.plence@inserm.fr); [ympers2000@yahoo.fr](mailto:ympers2000@yahoo.fr);  
[christian.jorgensen@inserm.fr](mailto:christian.jorgensen@inserm.fr); [florence.apparilly@inserm.fr](mailto:florence.apparilly@inserm.fr)

### Author contributions:

J.P. and G.C. designed and performed research, analyzed data and wrote the paper.

P.L-P. performed research, analyzed data and wrote the paper.

YMP, HY, JP, V.E., and D.S. contributed new reagents/analytical tools and wrote the paper.

D.K., S.G., C.J., and F.A. designed research and wrote the paper.

## **ACKNOWLEDGMENTS**

This work was supported by grants from the INSERM, the European community (Autocure LSHB-CT-2006-018661, EuroTraps HEALTH-F2-2008-200923), the “Société française de rhumatologie” (SFR), the University of Montpellier I, the “Fondation pour la Recherche Médicale” (FRM) and the Swiss National Fund for Research (SNF 32-120702/1). We would like to thank the animal facility staff from the INM (Institut des Neurosciences de Montpellier). We are also grateful to Meryem Ammari and Isabelle Duroux-Richard for technical help.

## REFERENCES

1. **Kinne RW**, Stuhlmüller B, Burmester GR. Cells of the synovium in rheumatoid arthritis. Macrophages. *Arthritis Res Ther* 2007; **9**:224.
2. **Tak PP**, Kalden JR. Advances in rheumatology: new targeted therapeutics. *Arthritis Res Ther* 2011; **13**:S5.
3. **Hamilton JA**, Tak PP. The dynamics of macrophage lineage populations in inflammatory and autoimmune diseases. *Arthritis Rheum* 2009; **60**:1210-21.
4. **Kimura A**, Naka T, Kishimoto T. IL-6-dependent and -independent pathways in the development of interleukin 17-producing T helper cells. *Proc Natl Acad Sci U S A* 2007; **104**:12099-104.
5. **Sato K**, Suematsu A, Okamoto K, *et al.* Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. *J Exp Med* 2006; **203**:2673-82.
6. **Sarkar S**, Cooney LA, Fox DA. The role of T helper type 17 cells in inflammatory arthritis. *Clin Exp Immunol* 2010; **159**:225-37.
7. **Tesmer LA**, Lundy SK, Sarkar S, *et al.* Th17 cells in human disease. *Immunol Rev* 2008; **223**:87-113.
8. **Thurlings RM**, Wijbrandts CA, Bennink RJ, *et al.* Monocyte scintigraphy in rheumatoid arthritis: the dynamics of monocyte migration in immune-mediated inflammatory disease. *PLoS One* 2009; **4**:e7865.
9. **Evans HG**, Gullick NJ, Kelly S, *et al.* In vivo activated monocytes from the site of inflammation in humans specifically promote Th17 responses. *Proc Natl Acad Sci U S A* 2009; **106**:6232-37.
10. **Wang T**, Zhang X, Bheda P, *et al.* Structure of Nampt/PBEF/visfatin, a mammalian NAD<sup>+</sup> biosynthetic enzyme. *Nat Struct Mol Biol* 2006; **13**:661-2.

11. **Zhang LQ**, Heruth DP, Ye SQ. Nicotinamide Phosphoribosyltransferase in Human Diseases. *J Bioanal Biomed* 2011; **3**:13-25.
12. **Samal B**, Sun Y, Stearns G, *et al.* Cloning and characterization of the cDNA encoding a novel human pre-B-cell colony-enhancing factor. *Mol Cell Biol* 1994; **14**:1431-37.
13. **Xie H**, Tang SY, Luo XH, *et al.* Insulin-like effects of visfatin on human osteoblasts. *Calcif Tissue Int* 2007; **80**:201-10.
14. **Rongvaux A**, Galli M, Denanglaire S, *et al.* Nicotinamide phosphoribosyl transferase/pre-B cell colony-enhancing factor/visfatin is required for lymphocyte development and cellular resistance to genotoxic stress. *J Immunol* 2008; **181**:4685-95.
15. **Schilling E**, Hauschildt S. Extracellular ATP induces P2X7-dependent nicotinamide phosphoribosyltransferase release in LPS-activated human monocytes. *Innate Immun* 2012; **18**.
16. **Luk T**, Malam Z, Marshall JC. Pre-B cell colony-enhancing factor (PBEF)/visfatin: a novel mediator of innate immunity. *J Leukoc Biol* 2008; **83**:804-16.
17. **Brentano F**, Schorr O, Ospelt C, *et al.* Pre-B cell colony-enhancing factor/visfatin, a new marker of inflammation in rheumatoid arthritis with proinflammatory and matrix-degrading activities. *Arthritis Rheum* 2007; **56**:2829-39.
18. **Schilling E**, Wehrhahn J, Klein C, *et al.* Inhibition of nicotinamide phosphoribosyltransferase modifies LPS-induced inflammatory responses of human monocytes. *Innate Immun* 2012; **18**:518-30.
19. **Moschen AR**, Kaser A, Enrich B, *et al.* Visfatin, an adipocytokine with proinflammatory and immunomodulating properties. *J Immunol* 2007; **178**:1748-58.



20. **Mesko B**, Poliska S, Szegedi A, *et al.* Peripheral blood gene expression patterns discriminate among chronic inflammatory diseases and healthy controls and identify novel targets. *BMC Med Genomics* 2010; **3**:1-15.
21. **Busso N**, Karababa M, Nobile M, *et al.* Pharmacological inhibition of nicotinamide phosphoribosyltransferase/visfatin enzymatic activity identifies a new inflammatory pathway linked to NAD. *PLoS One* 2008; **3**:e2267.
22. **Nowell MA**, Richards PJ, Fielding CA, *et al.* Regulation of pre-B cell colony-enhancing factor by STAT-3-dependent interleukin-6 trans-signaling: implications in the pathogenesis of rheumatoid arthritis. *Arthritis Rheum* 2006; **54**:2084-95.
23. **Evans L**, Williams AS, Hayes AJ, *et al.* Suppression of leukocyte infiltration and cartilage degradation by selective inhibition of pre-B cell colony-enhancing factor/visfatin/nicotinamide phosphoribosyltransferase: Apo866-mediated therapy in human fibroblasts and murine collagen-induced arthritis. *Arthritis Rheum* 2011; **63**:1866-77.
24. **Swirski FK**, Nahrendorf M, Etzrodt M, *et al.* Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science* 2009; **325**:612-6.
25. **Auffray C**, Sieweke MH, Geissmann F. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. *Annu Rev Immunol* 2009; **27**:669-92.
26. **Leuschner F**, Dutta P, Gorbatov R, *et al.* Therapeutic siRNA silencing in inflammatory monocytes in mice. *Nat Biotechnol* 2011; **29**:1005-10.
27. **Rhinn H**, Largeau C, Bigey P, *et al.* How to make siRNA lipoplexes efficient? Add a DNA cargo. *Biochim Biophys Acta* 2009; **1790**:219-30.
28. **Schlegel A**, Largeau C, Bigey P, *et al.* Anionic polymers for decreased toxicity and enhanced in vivo delivery of siRNA complexed with cationic liposomes. *J Control Release* 2011; **152**:393-401.

29. **Charbonnier LM**, Han WG, Quentin J, *et al.* Adoptive transfer of IL-10-secreting CD4<sup>+</sup>CD49b<sup>+</sup> regulatory T cells suppresses ongoing arthritis. *J Autoimmun* 2010; **34**:390-9.
30. **Charbonnier LM**, van Duivenvoorde LM, Apparailly F, *et al.* Immature dendritic cells suppress collagen-induced arthritis by in vivo expansion of CD49b<sup>+</sup> regulatory T cells. *J Immunol* 2006; **177**:3806-13.
31. **Courties G**, Baron M, Presumey J, *et al.* Cytosolic phospholipase A2 $\alpha$  gene silencing in the myeloid lineage alters development of Th1 responses and reduces disease severity in collagen-induced arthritis. *Arthritis Rheum* 2011; **63**:681-90.
32. **Khoury M**, Escriou V, Courties G, *et al.* Efficient suppression of murine arthritis by combined anticytokine small interfering RNA lipoplexes. *Arthritis Rheum* 2008; **58**:2356-67.
33. **Courties G**, Seiffart V, Presumey J, *et al.* In vivo RNAi-mediated silencing of TAK1 decreases inflammatory Th1 and Th17 cells through targeting of myeloid cells. *Blood* 2010; **116**:3505-16.
34. **Hoechst B**, Gamrekashvili J, Manns MP, *et al.* Plasticity of human Th17 cells and iTregs is orchestrated by different subsets of myeloid cells. *Blood* 2011; **117**:6532-41.
35. **Smeekens SP**, van de Veerdonk FL, Joosten LA, *et al.* The classical CD14<sup>++</sup> CD16<sup>-</sup> monocytes, but not the patrolling CD14<sup>+</sup> CD16<sup>+</sup> monocytes, promote Th17 responses to *Candida albicans*. *Eur J Immunol* 2011; **41**:2915-24.
36. **Li Y**, Zhang Y, Dorweiler B, *et al.* Extracellular Nampt promotes macrophage survival via a nonenzymatic interleukin-6/STAT3 signaling mechanism. *J Biol Chem* 2008; **283**:34833-43.
37. **Laudes M**, Oberhauser F, Schulte DM, *et al.* Visfatin/PBEF/Nampt and resistin expressions in circulating blood monocytes are differentially related to obesity and type 2 diabetes in humans. *Horm Metab Res* 2010; **42**:268-273.

38. **Fujimoto M**, Serada S, Mihara M, *et al.* Interleukin-6 blockade suppresses autoimmune arthritis in mice by the inhibition of inflammatory Th17 responses. *Arthritis Rheum* 2008; **58**:3710-9.
39. **Vandooren B**, Noordenbos T, Ambarus C, *et al.* Absence of a classically activated macrophage cytokine signature in peripheral spondylarthritis, including psoriatic arthritis. *Arthritis Rheum* 2009; **60**:966-75.
40. **Herenius MM**, Thurlings RM, Wijbrandts CA, *et al.* Monocyte migration to the synovium in rheumatoid arthritis patients treated with adalimumab. *Ann Rheum Dis* 2011; **70**:1160-2.
41. **Tak PP**, Smeets TJ, Daha MR, *et al.* Analysis of the synovial cell infiltrate in early rheumatoid synovial tissue in relation to local disease activity. *Arthritis Rheum* 1997; **40**:217-25.
42. **Zhao Q**. Dual targeting of CCR2 and CCR5: therapeutic potential for immunologic and cardiovascular diseases. *J Leukoc Biol* 2010; **88**:41-55.
43. **Lebre MC**, Vergunst CE, Choi IY, *et al.* Why CCR2 and CCR5 blockade failed and why CCR1 blockade might still be effective in the treatment of rheumatoid arthritis. *PLoS One* 2011; **6**:e21772.
44. **Geissmann F**, Manz MG, Jung S, *et al.* Development of monocytes, macrophages, and dendritic cells. *Science* 2010; **327**:656-61.
45. **King IL**, Dickendesher TL, Segal BM. Circulating Ly-6C<sup>+</sup> myeloid precursors migrate to the CNS and play a pathogenic role during autoimmune demyelinating disease. *Blood* 2009; **113**:3190-7.

46. **Nahrendorf M**, Swirski FK, Aikawa E, *et al.* The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *J Exp Med* 2007; **204**:3037-47.